Plant Physiol. (1996) 112: 537–547

The Tomato *E8* Gene Influences Ethylene Biosynthesis in Fruit but Not in Flowers[1]

Michelle L. Kneissl and Jill Deikman*

Department of Biology, 208 Mueller Laboratory, Biotechnology Institute, and Intercollege Graduate Program in Plant Physiology, Pennsylvania State University, University Park, Pennsylvania 16802

We investigated the function of the tomato (*Lycopersicon esculentum*) *E8* gene. Previous experiments in which antisense suppression of *E8* was used suggested that the E8 protein has a negative effect on ethylene evolution in fruit. E8 is expressed in flowers as well as in fruit, and its expression is high in anthers. We introduced a cauliflower mosaic virus *35S-E8* gene into tomato plants and obtained plants with overexpression of *E8* and plants in which *E8* expression was suppressed due to co-suppression. Overexpression of *E8* in unripe fruit did not affect the level of ethylene evolution during fruit ripening; however, reduction of E8 protein by co-suppression did lead to elevated levels during ripening. Levels for ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), and ACC oxidase mRNA were increased approximately 7-fold in fruit of plants with reduced E8 protein. Levels of *ACC synthase 2* mRNA were increased 2.5-fold, and *ACC synthase 4* mRNA was not affected. Reduction of E8 protein in anthers did not affect the accumulation of ACC or of mRNAs encoding enzymes involved in ethylene biosynthesis. Our results suggest that the product of the E8 reaction participates in feedback regulation of ethylene biosynthesis during fruit ripening.

Ethylene is a gaseous plant hormone involved in specific developmental processes, as well as in response to many external stresses. Ethylene biosynthesis is increased in response to stimuli such as wounding, pathogen attack, and drought (Abeles et al., 1992). During normal development, ethylene promotes a number of events, including senescence, seed germination, abscission, and fruit ripening (Abeles et al., 1992). In climacteric fruits such as tomatoes (*Lycopersicon esculentum*), bananas, and avocados, the initiation of ripening is associated with a burst in ethylene biosynthesis, accompanied by a large increase in the respiration rate (Rhodes, 1980). Tomato fruit ripening involves the autolysis of cell-wall pectins, the synthesis of lycopene and other carotenoid pigments, and changes in the acid and sugar content associated with taste (Gray et al., 1992). Ethylene biosynthesis during fruit ripening is autocatalytic, such that a small amount of ethylene stimulates a massive increase in ethylene production (Yang and Hoffman, 1984). In recent years, definitive evidence that ethylene controls fruit ripening has been obtained by suppression of ethylene production in transgenic tomato plants. These experiments resulted in fruit that failed to ripen or had severely retarded ripening (Hamilton et al., 1990; Oeller et al., 1991; Klee, 1993; Picton et al., 1993; Theologis et al., 1993).

Ethylene biosynthesis begins with the conversion of Met to *S*-adenosylmethionine, catalyzed by *S*-adenosylmethionine synthase, followed by the formation of ACC by ACS. Finally, ACC is converted by ACO to ethylene (Yang and Hoffman, 1984). Genes encoding enzymes involved in ethylene biosynthesis have been cloned from a number of species (Kende, 1993). In tomato, nine *ACS* genes have been identified (Zarembinski and Theologis, 1994), but only two of these genes, *LE-ACS2* and *LE-ACS4*, are expressed at a high level in fruit (Rottmann et al., 1991; Yip et al., 1992). There is a large amount of sequence divergence among the different *ACS* genes, and even within tomato, the sequence identity of ACS polypeptides to one another varies between 50 and 96% (Rottmann et al., 1991). ACO is encoded by a smaller gene family, and its members are more similar to one another. In tomato, there are three *ACO* genes, but only one is expressed in fruit (Holdsworth et al., 1988). ACC can also be converted to 1-(malonylamino)cyclopropane-1-carboxylic acid by ACC *N*-malonyl transferase (Kende, 1993). This reaction is usually not reversible and malonylation of ACC may contribute to control of the rate of ethylene biosynthesis. ACC *N*-malonyl transferase has been purified from tomato fruit and was shown to be a 38-kD monomer. Its activity is greatest in the pericarp of orange fruit and is induced by ethylene treatment of unripe fruit (Martin and Saftner, 1995).

E8 is a gene regulated by ethylene during tomato fruit ripening and has been shown to have a negative effect on ethylene biosynthesis (Peñarrubia et al., 1992). When the level of E8 protein was reduced in transgenic tomato plants by expression of an antisense *E8* gene, ethylene levels were increased 2- to 6-fold in ripening fruit. It is interesting that the predicted E8 amino acid sequence has significant homology with ACO. E8 and ACO share 34% amino acid sequence identity over 295 residues (Deikman and Fischer, 1988). Both E8 and ACO are homologous to a family of enzymes known as 2-oxoglutarate-dependent dioxygenases (Prescott, 1993). These enzymes require ferrous iron and a reducing agent (usually ascorbate) for activity in vitro, and most use 2-oxoglutarate and molecular oxygen

[1] This work was supported by the Cooperative State Research Service, U.S. Department of Agriculture, under agreement no. 94–37304–0954.

* Corresponding author; e-mail jxd14@psu.edu; fax 1–814–865–9131.

Abbreviations: ACO, ACC oxidase; ACS, ACC synthase; CaMV 35S, cauliflower mosaic virus 35S; LUC, luciferase; PG, polygalacturonase.

as co-substrates (ACO does not use 2-oxoglutarate; Prescott, 1993). Both ACO and E8 amino acid sequences contain regions predicted to form an amphipathic helix containing multiple Leu residues on one face (Peck et al., 1992; Kende, 1993). This structure indicates possible protein-protein interactions (Landschulz et al., 1988) and suggests that E8 function may involve interaction with another protein or the formation of a homodimer. Recent crystallization of isopenicillin *N* synthase, also a 2-oxoglutarate-dependent dioxygenase, suggests that the active site is buried within a conserved jelly-roll motif, which is present in ACO and possibly E8, forming a new structural family of enzymes (Roach et al., 1995).

E8 is transcriptionally activated at the onset of ripening (Lincoln and Fischer, 1988a). Analysis of *E8* expression in fruit of wild-type, mutant, and transgenic plants defective in ethylene biosynthesis and in fruit treated with an inhibitor of ethylene action indicated that *E8* is controlled in fruit by both ethylene and ethylene-independent fruit- ripening signals (Lincoln et al., 1987; Lincoln and Fischer, 1988a, 1988b; DellaPenna et al., 1989; Theologis et al., 1993). Analysis of the *E8* promoter revealed that separate *cis*-elements are involved in ethylene-responsive expression and in expression in response to the ethylene-independent fruit-ripening signals (Deikman et al., 1992). *E8* is not activated by ethylene in leaves (Lincoln and Fischer, 1988a), and its expression has been considered fruit-specific. A more complete knowledge of the expression pattern of *E8* is important, since the *E8* promoter has been used for genetic engineering of fruit ripening (Giovannoni et al., 1989; Good et al., 1994).

Several hypotheses have been suggested for the function of the E8 protein. Autoinhibition of ethylene biosynthesis has been demonstrated in a number of instances (Yang and Hoffman, 1984). E8 may negatively regulate ethylene biosynthesis directly or indirectly. For example, it is conceivable that E8 could directly interact with ethylene biosynthetic enzymes to inhibit their activity or that E8 could encode an enzyme that metabolizes ACC. It is also possible that E8 action could indirectly trigger feedback regulation of ethylene biosynthesis. For example, plants impaired in ethylene perception have an increased rate of ethylene production (Atta-Aly et al., 1987; Guzman and Ecker, 1990; Chi et al., 1991), and it is inferred that ethylene biosynthesis in such plants is up-regulated in an attempt to correct a perceived deficiency in ethylene levels. It has been hypothesized that E8 function could be required for ethylene perception, by oxidation of the putative metalloprotein ethylene receptor (Theologis, 1992).

To further understand the function of *E8*, we used transgenic plants to (a) localize *E8* expression at the tissue level to determine whether its expression is correlated with ethylene biosynthesis and (b) examine the effect of altering *E8* gene expression on the expression of genes encoding ethylene biosynthetic enzymes. We found that the expression of *E8* is not limited to tomato fruits but is also present in flowers. We also found that in fruit reduction in E8 protein resulted in an increase in the concentrations of ACO and *ACS2* mRNAs, but reduction in E8 protein does not appear to affect ethylene biosynthesis in anthers.

MATERIALS AND METHODS

Tomato (*Lycopersicon esculentum* cv Ailsa Craig) plants were grown under standard greenhouse conditions. Seeds for the *Never-ripe* mutant and background *L. esculentum* cv Rutgers were obtained from the C.M. Rick Tomato Genetics Resource Center (University of California, Davis). *E8* antisense seeds (line 125–19) were generously provided by Dr. Lola Peñarrubia (Peñarrubia et al., 1992). Seeds for transgenic tomato plants (cv UC82B) bearing an *E8-GUS* chimeric gene were obtained from Dr. Harry Klee. The *E8-GUS* gene includes *E8* 5′ flanking sequences from an *Eco*RI site at −2181 to an *Nco*I site introduced at the start of translation (Giovannoni et al., 1989). The *GUS*-coding sequences and vector were as described previously (Jefferson, 1987), as was the determination of the developmental stage of fruit (Lincoln et al., 1987). The developmental stage of flowers was based on the report of Ursin et al. (1989), with modifications to account for cultivar differences. Stage 1 was defined by sepal tips pulling apart slightly and a tip-to-pedicle length of 6 mm. Stage 2 flowers were those 10 mm in length, and stage 3 (green petal) and 4 (mature) flowers were as described previously (Ursin et al., 1989).

Construction of Chimeric Genes

To make the *E8-LUC* chimeric gene the following four fragments were ligated: (a) the *E8* 5′ flanking sequences from the *Eco*RI site at −2181 to an *Nco*I site introduced at the start of translation (Giovannoni et al., 1989) and filled in with Klenow, (b) a *Bsm*I-*Sst*I *LUC* gene fragment (Ow et al., 1986), (c) a *Sac*I-*Eco*RI fragment containing the 3′ poly(A) addition sequence from the *NOS* gene (from pBI101.2; Clontech, Palo Alto, CA), and (d) pUC18 digested with *Eco*RI. The *E8-LUC* portion of this construct was released by digestion with *Sac*I. The pBI101 vector was prepared by digestion with *Sma*I, addition of *Sac*I linkers, and digestion with *Sac*I. The vector was then gel-purified and ligated with the *E8-LUC* fragment.

To generate a −90 CaMV *35S* promoter-*LUC* gene, CaMV *35S* promoter sequences from *Eco*RV to *Bam*HI (from pBI121, Clontech) were ligated to a *Bam*HI-*Sst*I *LUC* fragment (Ow et al., 1986), an *Sst*I-*Eco*RI fragment containing the 3′ poly(A) addition sequence from the *NOS* gene (from pBI101.2, Clontech), and pUC119-digested with *Eco*RI and *Sal*I (filled in). The −90 *35S-LUC* gene was released by digestion with *Hin*dIII and *Sac*I. A plant transformation binary vector was prepared by digestion of pBI121 with *Hin*dIII and *Sac*I and gel-purified. The −90 *35S-LUC* fragment was ligated with this pBI121 vector (minus the *35S-GUS* gene).

To overexpress the *E8* gene, a construct was made consisting of the CaMV *35S* promoter fused to the transcribed sequences of *E8*. To this end, pBI121 (Clontech) was digested with *Sma*I and *Eco*RI (releasing the GUS sequences), and the gel-purified vector was ligated with *E8* genomic sequences from *Xmn*I (+6) to *Eco*RI (+2286).

All DNA fragments used in cloning were purified on agarose gels with DEAE membranes (Schleicher & Schuell). Each construct was verified by double-stranded DNA sequence analysis.

Plant Transformation

Sterile cotyledon pieces were infected with *Agrobacterium tumefaciens* LBA4404 bearing plasmids containing the chimeric genes as described previously (Deikman and Fischer, 1988), except that tobacco feeder cells were not used. The presence of T-DNA in primary transformed plants and their progeny was determined either by Southern blot analysis (Deikman and Fischer, 1988) or by PCR as described by Konieczny and Ausubel (1993).

Analysis of Reporter Gene Activity

Fruit slices were stained for GUS activity using 5-bromo-4-chloro-3-indoyl glucuronide (Gold Biotechnology, St. Louis, MO) as described previously (Montgomery et al., 1993). Staining was allowed to proceed for 5 h at room temperature. Stage 4 flowers were vacuum-infiltrated for 1 min in 5-bromo-4-chloro-3-indoyl glucuronide buffer (Jefferson, 1987) and then incubated overnight at 37°C. LUC activity was determined as described before (Xu et al., 1996).

Determination of Ethylene Evolution and ACC Concentration

Fruits were picked at the breaker stage and stored at 28°C in the dark. Ethylene evolution was measured daily by placing individual fruits in sealed 250-mL containers and incubating them for 1 h at room temperature. A 1-mL gas sample was then removed from the container and ethylene concentration was determined by GC (model 5840A, Hewlett-Packard). Stage 4 anthers were flash-frozen in liquid nitrogen. Endogenous ACC levels were measured according to the method of Singh et al. (1992) and compared with a standard curve. For measurement of ACC concentration, fruit tissues were tested for ethylene evolution as described above, and pericarp tissue was flash frozen at the peak of evolution. Tissue was then ground in 200 mM $NaPO_4$ buffer, pH 8.0, at a ratio of 1 g of tissue to 2 mL of buffer. The extract was then filtered through cheesecloth and centrifuged at $1.5 \times 10^4 g$ for 10 min at room temperature. An aliquot was assayed for ACC using the method of Lizada and Yang (1979) and compared with a standard curve.

RNA Blot Analysis

RNA from anthers, leaves, and ovaries was isolated as described by Deikman and Hammer (1995). RNA from fruit was isolated as described by DellaPenna et al. (1986). Total RNA was denatured with formaldehyde, separated by electrophoresis on agarose-formaldehyde gels, blotted onto membranes (Genescreen, New England Nuclear), and hybridized with ^{32}P-labeled DNA as described by Sambrook et al. (1989). Prehybridization and hybridization were carried out at 42°C in a buffer containing 5× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4; Sambrook et al., 1989), 50% (v/v) formamide, 5× Denhardt's solution (Sambrook et al., 1989), 1% (w/v) SDS, and 100 μg mL^{-1} denatured salmon sperm DNA. After hybridization, the blots were washed at 55°C in 0.1× SSPE, 0.05% (w/v) sarcosine, and 0.01% (w/v) sodium PPi. Hybridizing probe DNA was removed from the blot by treatment for 10 min at 100°C with 1% (w/v) SDS in 10 mM Tris, pH 8.0, and 1 mM EDTA and then reprobed as described above. Relative intensity of probe hybridization of the various mRNAs was quantified using a blot analyzer (model 603 Betascope; Betagen, Waltham, MA). The significance of the difference in transcript levels between wild-type and transgenic samples was determined by Student's *t* test.

E4, E8, and PG cDNAs were obtained from Dr. Robert L. Fischer (DellaPenna et al., 1987; Lincoln et al., 1987). The ACO cDNA was generously provided by Dr. Hans Kende (Peck et al., 1992). *ACS2* and *ACS4* cDNAs were kindly provided by Dr. Athanasios Theologis (Rottmann et al., 1991). Specific probes for each *ACS* gene were prepared by isolation and labeling of the 3′ *Eco*RI fragment of each cDNA (Rottmann et al., 1991).

Immunoblot Analysis

Protein was extracted by homogenizing tissue in buffer containing 0.06 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 20% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromphenol blue at a ratio of 3 mL of buffer per gram of tissue. Cellular debris was removed by centrifugation at $1.5 \times 10^4 g$ for 10 min. Protein concentration was determined with the a protein assay dye reagent (Bio-Rad). The samples were denatured at 100°C for 3 min and resolved by polyacrylamide gel electrophoresis (Laemmli, 1970). Duplicate gels were electrophoresed and stained with Coomassie blue to verify equal loading. The proteins were transferred onto nitro- cellulose membranes (Schleicher & Schuell) using an electroblot apparatus (Trans-Blot Cell, Bio-Rad) in 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol at 100 V for 3 h. Transfer was verified using 0.1% (w/v) Ponceau red solution and 5% (v/v) acetic acid. The nitrocellulose blot was then reacted with mouse polyclonal anti-E8 antibody (Peñarrubia et al., 1992) and goat anti-mouse alkaline phosphatase conjugate (Sigma) as described previously (Sambrook et al., 1989).

RESULTS

Localization of *E8* Expression

Previous studies have shown that the accumulation of E8 mRNA increases at the onset of tomato fruit ripening and that *E8* expression is controlled at the transcriptional level (Lincoln and Fischer, 1988a). We examined the localization of *E8* expression using three plants independently transformed with a chimeric gene containing the full-length (2181 bp) *E8* promoter fused to the coding sequences for the GUS gene. Fruits from both untransformed and transformed plants were stained for GUS activity. We observed no blue staining in fruit from untransformed controls (data not shown). In unripe (mature green 1) fruit, GUS staining was detectable in vascular bundles but not in intervascular parenchyma cells (Fig. 1A). At the first sign of red pigment formation in the fruit, the mature green 4 (MG4) stage, the

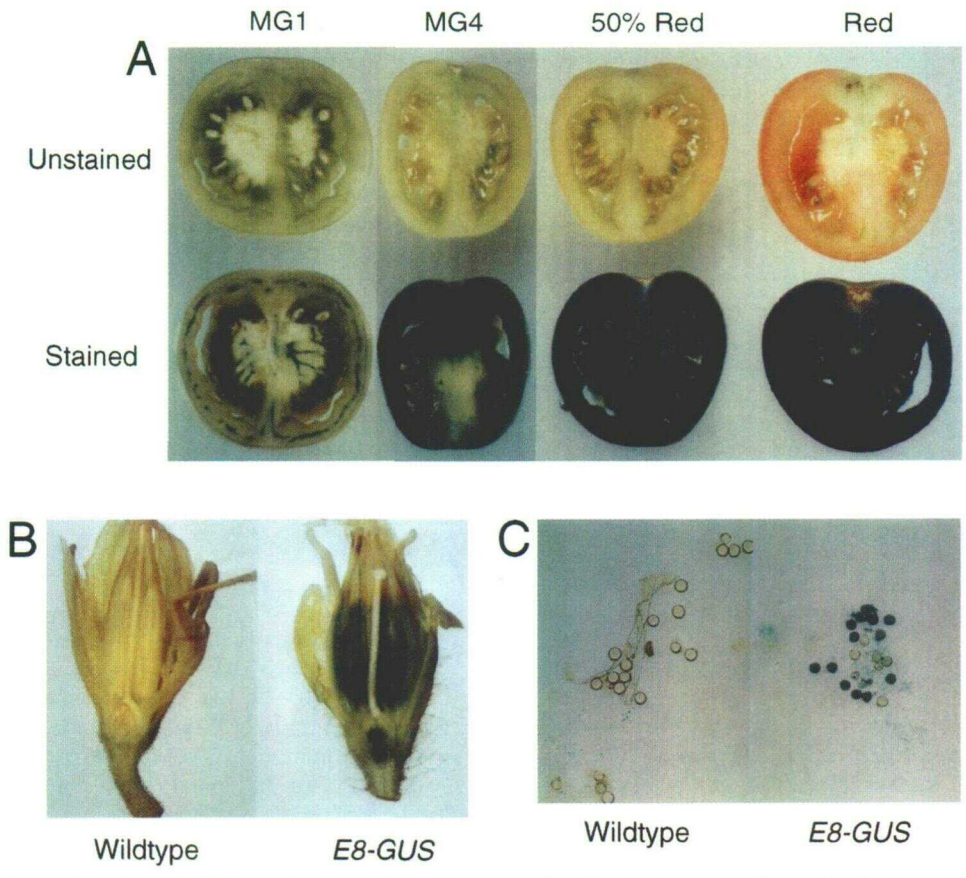

Figure 1. Expression of an *E8-GUS* gene in tomato fruits, flowers, and pollen. A, Fruits of different developmental stages from an *E8-GUS* transformant were sliced, and slices were photographed either unstained or stained for GUS activity. No GUS staining was evident in untransformed controls (not shown). B, Wild-type and *E8-GUS* stage 4 flowers were cut in half and then stained for GUS activity. C, Mature pollen grains of wild-type or *E8-GUS* flowers were stained for GUS activity. Notice the ratio of stained to unstained pollen grains in the *E8-GUS* pollen, indicating the hemizygous genotype of the parent.

pericarp stained darkly for GUS activity, but there was little staining in the columella. Expression of the *E8-GUS* gene continued to increase throughout ripening of the fruit including the columella tissue. *E8-GUS* expression at the red ripe stage was at a uniformly high level throughout the fruit.

Although *E8* expression had been considered to be fruit-specific, we also found significant *E8* expression in mature (stage 4) flowers (Fig. 1B). Flowers at this stage exhibited moderate levels of expression in the anther cone and low levels in the ovary. No GUS activity was found within the style or stigma (data not shown). We also found GUS activity in mature pollen grains of *E8-GUS* plants but not in pollen from untransformed control plants (Fig. 1C). Only about half of the pollen from this transgenic plant stained blue, indicating that the *E8-GUS* gene was active in the gametophytic stage of this plant, which was hemizygous for the transgene. Because of reports of artifactual GUS expression in pollen (Uknes et al., 1993), we also examined the expression of an *E8-LUC* gene in stably transformed plants. We found high levels of LUC activity in the pollen of plants transformed with *E8-LUC* and much lower levels of LUC activity in the pollen of plants transformed with a chimeric gene consisting of a minimal CaMV *35S* promoter fused to *LUC* (Fig. 2). These results confirm that the *E8* promoter is active in pollen.

To further define *E8* expression in flowers, we analyzed RNA extracted from anthers and ovaries of mature (stage 4) flowers. We found that there was a significant amount of E8 mRNA in the anthers and a low level in the ovaries (Fig. 3A). We also isolated proteins from anthers and ovaries of flowers of different stages and analyzed them by immunoblotting with an anti-E8 antibody (Fig. 3B). We found that E8 protein is only detectable in stage 4 anthers but not in the anthers of flowers at earlier stages of development. A low level of E8 protein was transiently detected in stage 3 ovaries. We were not able to detect E8 mRNA or protein in pollen isolated from mature flowers (data not shown). For this reason, we believe that the majority of the E8 protein present in stage 4 anthers accumulates in the anther wall.

To determine whether E8 protein is regulated by ethylene in anthers, as is the case in fruit, we examined the effect of the *Never-ripe* (*Nr*) mutation on accumulation of E8 protein in anthers. The *Nr* gene is thought to encode an ethylene receptor, and ethylene insensitivity of *Nr* mutant plants has been demonstrated both for fruit ripening and for several seedling growth parameters (Lanahan et al., 1994). Both transcription and mRNA accumulation of E8 is

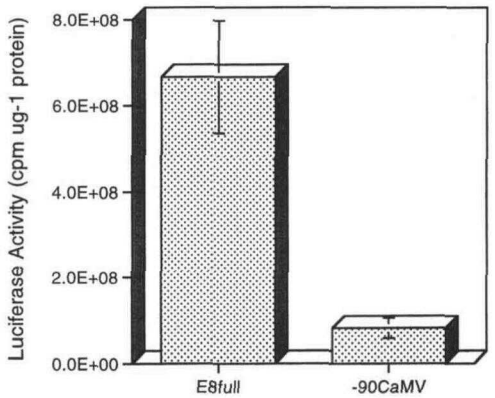

Figure 2. Luc activity in pollen of transgenic plants. E8full, Full-length *E8* promoter fused to *LUC*; −90CaMV, 90-bp CaMV *35S* promoter fused to *LUC*. $n = 5$, with pollen collected from two flowers per measurement. Measurements were pooled from three independent transformants for E8full and two independent transformants for −90CaMV. Error bars represent SEs.

significantly decreased in the fruit of *Nr* mutant plants (DellaPenna et al., 1989). In contrast, we found that a normal level of E8 protein accumulated in stage 4 anthers of *Nr* flowers, compared with a nearly isogenic control plant (Fig. 3C).

Overexpression of E8 Protein in Transgenic Tomato Plants

To learn more about the function of E8 in the fruits and flowers of tomato plants, we performed experiments to alter E8 protein levels in transgenic plants. We transformed plants with a chimeric gene consisting of the CaMV *35S* promoter fused to the transcribed sequences of the *E8* gene (Fig. 4A) and expected this highly active promoter to result in high levels of expression throughout the transgenic plants (Odell et al., 1985).

We analyzed *E8* expression in the fruit of 24 independently transformed plants by RNA gel blots (data not shown). Eighteen (75%) of these plants had greatly increased levels of E8 mRNA in unripe fruit, and six (25%) exhibited co-suppression (Napoli et al., 1990; van der Krol et al., 1990) and had greatly reduced levels of E8 mRNA in ripening fruit compared with untransformed control plants (data not shown). E8 protein levels corresponded to E8 mRNA levels in the fruit of transgenic plants. Immunoblot analysis of a few representative lines is shown in Figure 4B. Normally there is no detectable E8 protein in unripe fruit, but *E8*-overexpressing fruit had levels of E8 protein in unripe fruit similar to those found in ripening fruit (Fig. 4B). However, these plants did not contain a significantly higher concentration of E8 protein in their ripening fruit compared with wild-type plants. At most there was a doubling in the amount of E8 protein in the ripening fruit of the *E8*-overexpressing plants. Plants with *E8* overexpression in fruit had slightly increased levels of E8 protein in the anthers (Fig. 4B). In plants that exhibited co-suppression, E8 protein levels in both fruits and anthers were significantly reduced; we did not detect any E8 protein in fruit or anthers from these lines by immunoblotting. We also did not detect any E8 protein in fruit

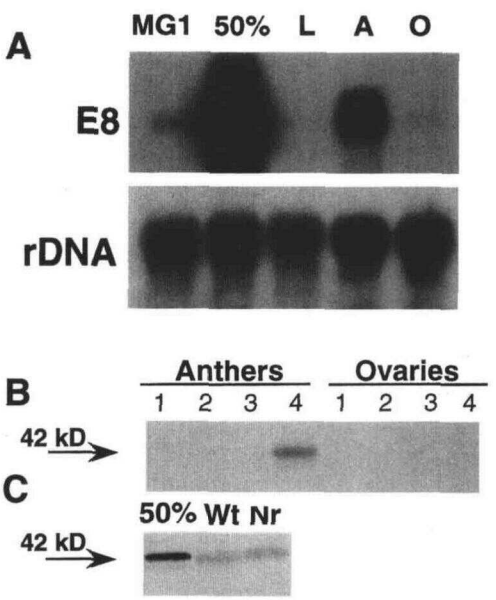

Figure 3. E8 expression in tomato flowers. A, RNA gel blot analysis comparing *E8* mRNA levels in fruit (MG1 = mature green 1; 50% = 50% red), leaves (L), anthers (A), and ovaries (O), from stage 4 flowers; 10 μg of total RNA was loaded per lane. B, Immunoblot of E8 protein in extracts from anthers and ovaries of stage 1 to 4 flowers (lanes 1–4). C, Immunoblot of E8 protein in extracts from wild-type (Wt) compared with *Nr* (Nr) stage 4 anthers. For immunoblots 7 μg of protein from anthers and ovaries and 1.4 μg of protein from fruit was loaded on the gel.

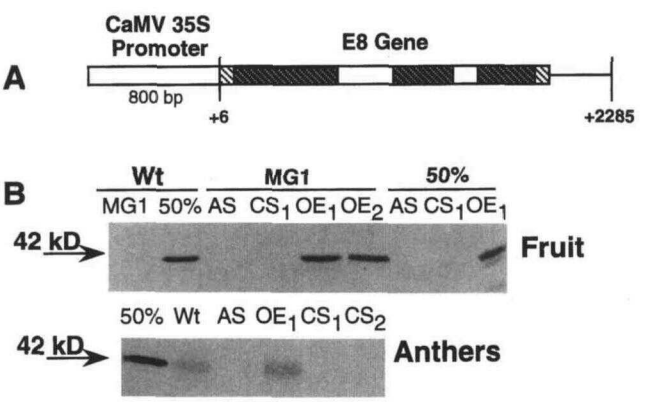

Figure 4. Altered E8 protein levels in transgenic tomato plants. A, Chimeric gene introduced into plants to achieve ectopic expression of *E8*. The 800-bp CaMV 35S promoter was fused to the transcribed sequences of the E8 gene from +6 to +2285 bp from the transcription initiation site. Hatched boxes represent exons, and lighter hatching indicates untranslated leader sequences. B, Immunoblot of E8 protein in extracts from fruits and anthers of plants transformed with the *35S-E8* gene or with an *E8*-antisense construct (Peñarrubia et al., 1992). Wt, Wild type; MG1, mature green 1 fruit; 50%, 50% red fruit; AS, *E8* antisense, CS, co-suppressed; OE, overexpressed. Subscripts indicate independent transformants. For fruit samples 1 μg of protein and for anthers 7 μg of protein was loaded on the gel.

or anthers from plants bearing an E8-antisense construct (Peñarrubia et al., 1992; Fig. 4B).

We determined the number of transgenes present in each individual transformant by Southern analysis (data not shown) and selected six individuals with insertions at a single locus for further analysis. Three of these lines were *E8* overexpressers, and three had reduced E8 protein.

Effect of E8 Protein Levels on Ethylene Evolution and Biosynthesis

When E8 protein levels were reduced by antisense expression of the *E8* gene, the amount of ethylene produced during fruit ripening was increased (Peñarrubia et al., 1992). We found that lines with reduced E8 protein levels due to co-suppression exhibited the same phenomenon and that fruit of both *E8* antisense and co-suppressed plants had 7- to 8-fold more ethylene than the wild type at the peak of ethylene evolution (Fig. 5A). However, we found that neither the substantial increase in E8 protein level at the MG1 stage nor the 2-fold increase in ripening fruit had any significant effect on ethylene evolution in ripening tomato fruits (Fig. 5A).

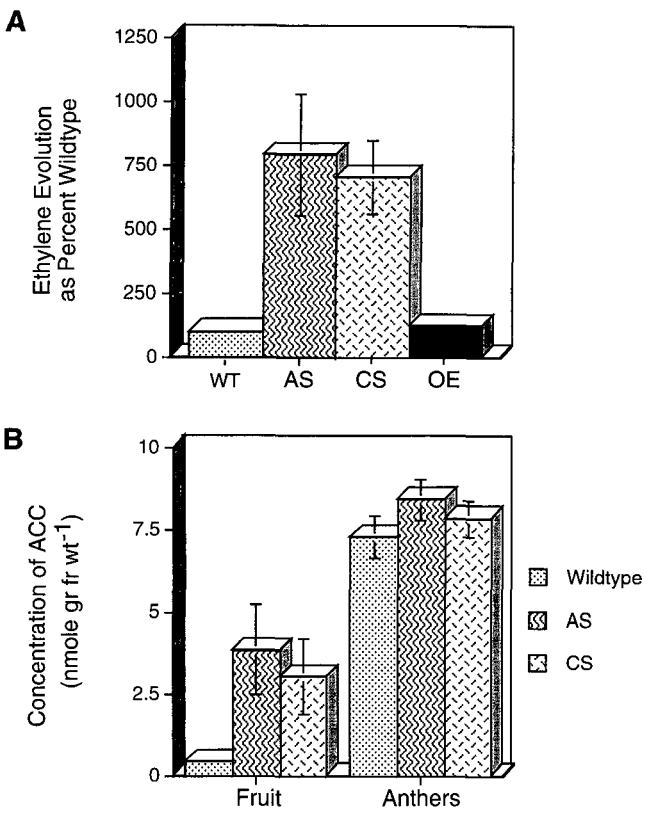

Figure 5. Ethylene evolution in fruits and ACC levels in anthers and fruits of wild-type and transgenic tomato plants. A, Peak ethylene evolution in fruits of wild-type and transgenic plants. WT, Wild type; AS, *E8* antisense; CS, co-suppressed; and OE, overexpressed. n = 9 to 16. B, Levels of ACC in fruits and anthers of transgenic and wild-type plants. Fruits were flash-frozen at the peak of ethylene evolution, n = 3. Stage 4 anthers, n = 6 to 9, with two anthers per measurement. Error bars represent SES. Where bars are not shown the error was no greater than the size of the symbol. gr fr wt, Grams fresh weight.

To further investigate the effect that altering E8 protein levels has on ethylene evolution, we measured the levels of ACC, the immediate precursor to ethylene, in both stage 4 anthers and ripening fruits of our transgenic plants. We found that the fruit of *E8* co-suppressed and antisense plants exhibited an approximately 7- to 9-fold increase, respectively, in ACC levels compared with wild type at the peak of ethylene evolution (Fig. 5B). Thus, the increase in ACC levels corresponded well with the increase in ethylene evolution measured in fruits of these lines (Fig. 5A). We were not able to measure ethylene evolution from flowers because of the induction of wound ethylene from picked flowers and because of the short life span of the picked flowers. We did measure ACC from isolated anthers, which were flash-frozen immediately after harvest. In contrast to what we found in fruit, reduction in E8 protein had no effect on endogenous ACC levels of stage 4 anthers (Fig. 5B).

To examine the mechanism by which E8 protein influences ethylene biosynthesis in ripening fruit, we measured the relative levels of mRNAs encoding the enzymes involved in ethylene biosynthesis. RNA was isolated from individual fruits harvested at the first sign of red color formation, and then frozen 2 d later, when ethylene evolution was maximal. We examined mRNA in fruit from wild-type plants, the *E8* antisense plant, and two independently transformed plants that had co-suppression of *E8*. The level of E8 mRNA was reduced approximately 20-fold in both *E8* antisense and co-suppressed fruit compared with wild type (Fig. 6). This number is most likely an underestimate of the amount of reduction of the E8 message, because it includes a signal from a band of unknown identity that hybridized to the E8 probe and migrated just above the E8 mRNA but that was not affected by antisense or co-suppression of the *E8* gene. ACO mRNA levels were significantly increased (approximately 6.6-fold [Fig. 6]) in fruits with reduced levels of E8 protein. Gene-specific probes for both *ACS2* and *ACS4* were used to examine ACS mRNA levels. *ACS2* mRNA concentration increased moderately (about 2.5-fold [Fig. 6]) in fruits with reduced levels of E8 mRNA. The accumulation of *ACS4* mRNA was highly variable among individual fruits of both wild-type and transgenic plants, and reducing E8 protein concentration had no effect on *ACS4* mRNA levels (data not shown). The mRNA for PG was also not significantly affected by a reduction in E8 protein concentration (Fig. 6).

To assess the sensitivity to ethylene of plants with reduced E8 protein, we examined the accumulation of mRNA for the ethylene-responsive *E4* gene (Fig. 6). We found that E4 mRNA accumulated in fruit to a level more than twice that of wild type in plants with reduced E8 protein.

We also examined the expression of genes of the ethylene biosynthetic pathway in anthers from plants with reduced E8 protein. We isolated RNA from approximately 10 stage 4 anthers from wild-type plants, a co-suppressed line, and the antisense line. The level of E8 mRNA in stage 4 anthers

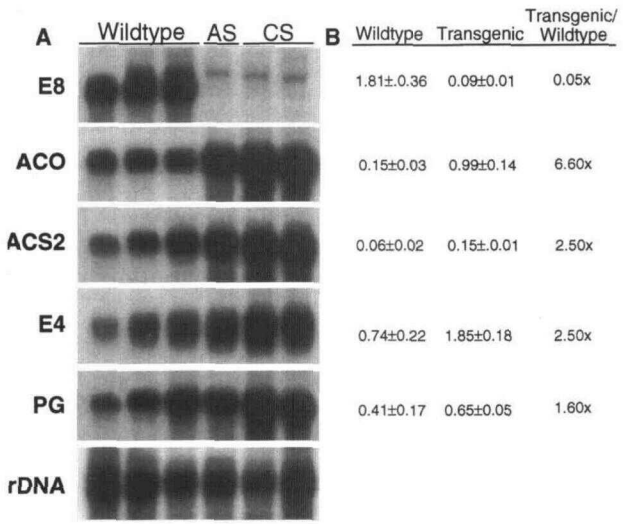

Figure 6. Accumulation of specific mRNAs in fruit with reduced E8 protein. A, RNA gel blot analysis of RNA from individual wild-type and transgenic tomato fruits. Fruits were flash-frozen at the peak of ethylene evolution. Total RNA (18 μg) from individual fruits was loaded in each lane. AS, Antisense; CS, co-suppressed. B, Relative concentration of each transcript in fruit from wild-type and transgenic plants. Each band was quantified using a blot analyzer (Betascope, Betagen) and corrected for differences in loading by dividing with counts per minute for rDNA hybridization. The average of the three replicas is shown (cpm ± SE). The difference in transcript levels between wild-type and transgenic fruit was significant, with $P > 0.95$ except for the PG mRNA.

was reduced approximately 9-fold (Fig. 7) in these lines. In anthers, mRNA levels for ACO and *ACS2* were not affected by the concentration of E8 protein (Fig. 7). The level of *ACS2* expression in wild-type plants was different in the two samples harvested several weeks apart. However, the amount of *ACS2* mRNA in the anthers was similar in the transgenic plant and the untransformed control that was harvested at the same time. No expression of *ACS4* was detected in the anthers of any of the genotypes tested (data not shown). We also found that the *E4* gene was expressed in stage 4 anthers, and its expression was also not affected by the level of E8 protein in the anthers (Fig. 7).

To determine whether the changes in accumulation of ACO and *ACS2* mRNAs in the fruit of transgenic plants with reduced E8 protein could be a response to the increased levels of endogenous ethylene, we examined the accumulation of these transcripts in wild-type, unripe fruit treated with air or with 20 μL/L ethylene for 8 h. Figure 8 shows that ACO and E4 mRNA levels were both increased by a similar amount (26- and 20-fold, respectively) in fruit treated with ethylene. In contrast, mRNA concentrations for *ACS2* and *ACS4* were not affected by an 8-h treatment with ethylene, although the levels of these transcripts did increase during fruit ripening (Fig. 8).

DISCUSSION

E8 Affects Ethylene Biosynthesis during Fruit Ripening

The reduction of E8 protein by antisense expression of *E8* in tomato plants was previously shown to result in an increase in the level of ethylene produced during fruit ripening (Peñarrubia et al., 1992). We have now reproduced that effect by a different method of reducing E8 protein levels. We found that in plants exhibiting co-suppression of *E8* there was a similar increase in the ethylene evolution rate in ripening fruit (Fig. 5A). Ethylene has been shown to regulate its own biosynthesis, both positively and negatively (Yang and Hoffman, 1984). Autoinhibition of ethylene biosynthesis has been demonstrated by application of exogenous ethylene to wounded flavedo tissue of citrus fruits (Riov and Yang, 1982), to banana fruits (Vendrell and McGlasson, 1971), and to sycomore figs (Zeroni and Galil, 1976). It is possible that the increase in ethylene evolution demonstrated in both *E8* co-suppressed and antisense lines is due to the loss of negative regulation of ethylene biosynthesis with the loss of E8 function. This effect could result from a direct interaction of E8 with enzymes in the ethylene biosynthetic pathway. Alternatively, the effect could be indirect. For example, the product of the E8 reaction could influence a signal transduction pathway that regulates ethylene biosynthesis.

E8 Effect on Ethylene Biosynthesis Is Specific to Fruit Ripening

Our discovery of *E8* expression in flowers allowed us to compare the effects of reducing E8 protein levels in two different organs. If E8 affects ethylene biosynthesis directly, we should see the same effect on ethylene evolution in flowers that we see in fruit. We focused on stage 4 anthers because that was the part and stage of the flower that had the greatest amount of E8 mRNA and protein (Figs. 1 and 3). We were not able to detect any E8 protein

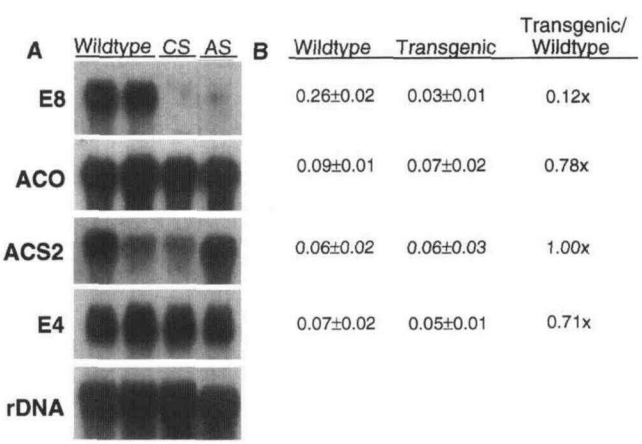

Figure 7. Accumulation of specific mRNAs in tomato anthers with reduced E8 protein. A, RNA gel blot analysis of RNA from transgenic stage 4 anthers. Total RNA (18 μg) pooled from approximately 10 anthers was loaded in each lane. AS, Antisense; CS, co-suppressed. Anthers for the wild type shown in lane 1 were harvested at the same time as the antisense anthers, and anthers for the wild type shown in lane 2 were harvested at the same time as the co-suppressed anthers. B, Relative concentration of each transcript in anthers from wild-type and transgenic plants. Each band was quantified as described for Figure 6. The average of the two replicas is shown (cpm ± SE).

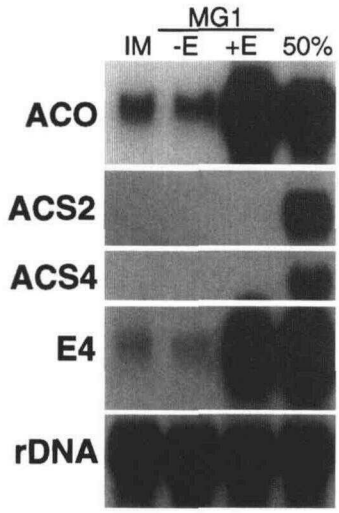

Figure 8. Accumulation of specific mRNAs in response to ethylene. RNA was isolated from immature fruit (IM), unripe (MG1) fruit treated with air (−E) or ethylene (+E), and 50% red fruit of wild-type plants. Each lane contained 20 μg of RNA.

in anthers from our plants with co-suppression or antisense suppression of *E8* by immunoblotting, even after extended developing times. Furthermore, we analyzed anthers from the same plants that exhibited an ethylene overproduction phenotype in the fruit. We did not directly measure ethylene evolution from anthers because we expected wound ethylene production from excised tissue (Abeles et al., 1992), which would prevent an assessment of ethylene levels in intact anthers. Instead, we measured ACC levels in anthers from wild-type plants and transgenic plants with reduced E8 protein. Although ACC levels in fruit increased to the same degree as ethylene levels, we found that ACC levels in anthers were not affected by E8 protein concentration (Fig. 5B). Furthermore, whereas levels for ACO and *ACS2* mRNA were increased by a reduction in E8 protein in fruit, levels of these mRNAs were not affected in the anthers of these same transgenic plants. These data suggest that E8 does not function primarily as a regulator of ethylene biosynthesis.

We did not see an effect from overproduction of E8 protein on ethylene evolution in fruit. In plants overexpressing the E8 protein, we were able to achieve only an approximately 2-fold increase in E8 protein concentration during fruit ripening, but a 2-fold difference in ethylene evolution would be resolvable (Peñarrubia et al., 1992). It is possible that the E8 protein is normally present at saturating levels in ripening fruits. In fact, Peñarrubia et al. (1992) found that ethylene overproduction was present only in fruit in which E8 protein was reduced to levels that were undetectable by immunoblotting. Furthermore, we were able to greatly increase the level of E8 protein in unripe fruit and obtained plants with unripe fruit that had the same amount of E8 protein as is normally present in a ripening fruit (Fig. 4B). However, this massive increase of E8 in unripe fruit did not affect ethylene production during fruit ripening (Fig. 5A). These results indicate that *E8* does not itself encode the enzyme that metabolizes ACC, ACC *N*-malonyl transferase, because, if *E8* encoded malonyl transferase, overproduction of E8 protein at the unripe and ripening stages would reduce ACC pools and, therefore, ethylene levels during ripening.

The fact that the E8 effect is specific to ripening fruit and that an excess of E8 protein has no measurable effect on ethylene biosynthesis suggests that E8 does not directly interact with enzymes involved in ethylene biosynthesis. It also supports the idea that E8 acts in conjunction with another factor present only in ripening fruit to affect ethylene biosynthesis.

E8 Affects Multiple Steps of Ethylene Biosynthesis

We have carried out experiments to identify steps in ethylene biosynthesis that are affected by reductions in E8 protein. We determined that ACC, the direct precursor to ethylene, is increased to a similar degree as ethylene in fruit from transgenic plants (Fig. 5B). We also found that the concentrations of mRNAs of two genes in the ethylene biosynthetic pathway, *ACO* and *ACS2*, are increased by the reduction in E8 protein (Fig. 6). ACO mRNA was increased 6.6-fold and *ACS2* mRNA was increased 2.5-fold. We found no effect of E8 on accumulation of *ACS4* mRNA (data not shown).

The amount of increase in ACO mRNA correlated well with the increase in ethylene evolution. In fact, ACC, ACO mRNA, and ethylene evolution were all increased approximately 7- to 9-fold in fruits with reduced E8 protein levels. The 2.5-fold increase in *ACS2* message is not sufficient to account for the increase in ACC levels that we measured. It is possible that an *ACS* gene not normally expressed during fruit ripening is activated in the transgenic plants. Alternatively, reduction in E8 protein may affect the activity or stability of the ACS protein. ACS has a relatively short half-life, approximately 40 to 58 min in wounded tomato fruit tissue (Kende and Boller, 1981; Kim and Yang, 1992). Thus, control of the stability of the ACS enzyme could be a means of regulation of ethylene biosynthesis. The finding that the activity of ACS synthesized in bacteria is increased by deletion of 46 to 52 amino acids from the carboxy terminus demonstrates a possible mechanism for control of ACS activity (Li and Mattoo, 1994).

To determine whether the increases in ACO and *ACS2* mRNAs that we observed in fruit with reduced E8 protein could be due to an increase in endogenous ethylene levels, we examined the accumulation of these mRNAs in response to an 8-h treatment of unripe, wild-type fruit with ethylene. Under these conditions, mRNAs for the *E4* and *E8* genes accumulate to high levels, but mRNAs for some genes regulated during fruit ripening, such as PG, do not increase (Lincoln et al., 1987). We showed that ACO mRNA concentration in unripe fruit increased within 8 h of ethylene treatment to levels similar to E4 mRNA. However, ACO mRNA was increased 6.6-fold in the transgenic fruit, whereas E4 mRNA was increased only 2.5-fold. If both mRNAs were responding only to endogenous ethylene in the transgenic fruit, one might expect that their levels would increase by similar amounts. Since ACO mRNA levels increased much more than E4 mRNA levels, ACO

mRNA accumulation in fruit from the transgenic plants could be responding to other signals in addition to ethylene. Similar reasoning can be used to infer that *ACS2* mRNA levels in the transgenic fruit may increase in response to signals other than ethylene. *ACS2* mRNA accumulation was not rapidly induced by ethylene (Fig. 8), although it did accumulate in response to physiological levels of ethylene after 48 h of treatment (Rottmann et al., 1991). Regulation of *ACS2* expression during fruit ripening thus appears similar to the PG gene (Gray et al., 1992). Transgenic plants with reduced E8 protein did not accumulate significantly more PG mRNA than wild-type plants (Fig. 6), suggesting that the increase in *ACS2* mRNA that we observed could be due to signals in addition to ethylene.

Taken together, our results indicate that E8 does not affect one single step of the ethylene biosynthetic pathway, but rather affects several steps, and that E8 action may influence a regulatory pathway active only in ripening fruit, which controls ethylene biosynthesis. One way that E8 could indirectly affect ethylene evolution is for it to be involved in ethylene perception (Theologis, 1992). However, we found that the ethylene-responsive *E4* gene is expressed at normal levels in plants with reduced levels of E8 protein (Fig. 6). In fact, E4 mRNA levels are increased about 2.5-fold in the fruit of plants with reduced E8 protein, probably in response to the increase in ethylene production in these fruit. This result indicates that ethylene perception is intact in plants with reduced E8 protein.

Variability in ACS mRNA Levels in Wild-Type Plants

We observed a considerable amount of variability in the level of *ACS4* mRNA among individual fruits of both wild-type and transgenic plants when mRNA levels were normalized to the amount of rRNA present in the sample (not shown). In contrast, levels of *ACO* and *E8* mRNAs were fairly constant from fruit to fruit (Fig. 6). Reduction in E8 protein had no effect on *ACS4* mRNA accumulation. Our comparison of mRNAs in individual fruits has allowed us to observe variability in mRNA levels among individuals, which has not been previously reported. We also observed variability in the amount of *ACS2* mRNA in wild-type anthers harvested on different dates, whereas other mRNAs, including *ACO*, *E8*, and *E4*, were not affected by this seasonal difference (Fig. 7). The *ACS* genes are known to respond to a variety of internal, environmental, and chemical signals, such as wounding, Li^+, exogenously applied auxin, cytokinin, and ethylene (Kende, 1993), and may therefore be more sensitive to fluctuations in environmental parameters than the other genes we studied. The variability we have observed among individuals in *ACS* mRNA levels deserves further investigation.

E8 Expression in Flowers

We have found that *E8* is expressed in floral tissues as well as in the fruit and that its expression in flowers is developmentally regulated. *E8* is expressed in anthers only when they are mature (Fig. 3B), but *E8* is also transiently expressed at a low level in stage 3 ovaries. GUS activity in *E8-GUS* flowers did not correlate perfectly with data obtained by immunoblotting. For example, we detected GUS staining in stage 4 ovaries of a flower from an *E8-GUS* plant but only detected *E8* protein in stage 3 ovaries. It is possible that the GUS activity in stage 4 ovaries is due to a stable GUS protein produced during stage 3 (Jefferson et al., 1987). Also, although we detected both *E8-GUS* and *E8-LUC* expression in the pollen of transgenic plants (Fig. 1C), we were not able to detect E8 protein or mRNA (data not shown). The fact that GUS staining was observed only in approximately half of the *E8-GUS* pollen grains indicates that the *E8* gene is expressed in the gametophytic stage. Thus, the GUS protein is not synthesized in maternal tissue and imported into the pollen. *E8* may be expressed at a very low level in pollen so that we were not able to detect *E8* mRNA by blotting total RNA but were able to detect GUS activity because the stability of the protein allows accumulation to detectable levels and LUC activity because of the absence of endogenous LUC activity. It is also possible that *E8* mRNA and protein are produced prior to pollen maturation and that these products are not stable in mature pollen. In that case, the GUS and LUC activity we observed could be due to protein synthesized at this earlier time of pollen development. These proteins might have sufficient stability to be detectable in the mature pollen. Resolving the discrepancy between the expression of reporters and *E8* mRNA and protein will require a finer analysis of these products during pollen development, including the use of more sensitive techniques to measure mRNA and protein levels.

Although *E8* is ethylene-regulated in fruit, our results suggest that it may not be so regulated in anthers. In ethylene-insensitive *Nr* mutant plants, *E8* transcription and mRNA accumulation are significantly reduced in ripening fruits (DellaPenna et al., 1989). However, we found that E8 protein levels are normal in stage 4 anthers of *Nr* mutant tomato plants (Fig. 3C). The *Nr* locus has been cloned and shown to be a homolog of the Arabidopsis ethylene receptor *ETR* (Wilkinson et al., 1995). However, the ethylene receptor may be encoded by more than one gene (Hua et al., 1995), and a second tomato *ETR* homolog that is constitutively expressed has been identified (Theologis, 1996; Zhou et al., 1996). It is possible that a gene other than *NR* controls ethylene perception in the anthers of tomato.

E8 expression in anthers occurs at the same time as expression of *ACS2* in anthers (Rottmann et al., 1991). ACO is also known to be expressed in the anthers of other species (Tang et al., 1994), and we found significant levels of ACC in tomato anthers (Fig. 5B). The pollen of many species has been shown to contain extremely high levels of ACC (Whitehead et al., 1983). It is thought that in some species pollen-borne ACC is converted to ethylene upon pollination and that ethylene then acts as a signal that coordinates postpollination events (Whitehead et al., 1983; Singh et al., 1992). However, the fact that we did not find any effect on expression of ethylene biosynthesis genes in anthers after reducing E8 protein levels suggests that this coordinate expression of *E8* and genes encoding ethylene

biosynthesis enzymes may be coincidental. We should look to properties other than ethylene biosynthesis that anthers, pollen, and fruit have in common to direct future investigations of *E8* function.

CONCLUSIONS

E8 action has a significant negative effect on ethylene biosynthesis in fruit. In fruit, E8 appears to affect multiple steps of ethylene biosynthesis, suggesting that it influences a regulatory pathway that controls the rate of ethylene biosynthesis. Our results suggest that E8 does not function primarily to regulate ethylene biosynthesis, since it does not affect it in anthers the same way that it does in fruit. It is likely that E8 has the same primary function in both anthers and in fruit but that the product of the enzymatic reaction catalyzed by E8 affects only ethylene biosynthesis in fruit. Further study of the role of E8 in flowers and fruit is necessary for a complete understanding of the mechanisms for the control of ethylene biosynthesis.

ACKNOWLEDGMENTS

We acknowledge Anthony Panarello and Peggy Urban for excellent technical assistance. We thank Dr. Robert L. Fischer for providing anti-E8 antibody and E4, E8, and PG DNAs. We thank Drs. Lola Peñarrubia and Robert L. Fischer for *E8* antisense seed, Dr. Harry Klee for providing seeds for *E8-GUS* plants, Dr. Athanasios Theologis for ACS cDNA clones, and the C.M. Rick Tomato Genetics Resource Center for providing seeds of the *Nr* mutant. We are grateful to Dr. Hans Kende for the ACO cDNA clone and for advice concerning the measurement of ACC in fruit. We thank Dr. Kathy Evensen for generously allowing us to use her gas chromatograph and ethylene tank and for critically reading the manuscript. We acknowledge Laura Haubrick for staining *E8-GUS* pollen, Joe Ciardi for *E8-LUC* transformants, Jack Fowler for equipment manufacture, and Tony Omeis for excellent greenhouse services.

Received May 1, 1996; accepted June 29, 1996.

LITERATURE CITED

Abeles FB, Morgan PW, Saltveit ME Jr (1992) Ethylene in Plant Biology. Academic Press, San Diego, CA

Atta-Aly M, Saltveit ME Jr, Hobson GE (1987) Effect of silver ions on ethylene biosynthesis by tomato fruit tissue. Plant Physiol **83:** 44–48

Chi G-L, Pua E-C, Goh C-J (1991) Role of ethylene on *de novo* shoot regeneration from cotyledonary explants of *Brassica campestris* ssp. pekinensis (Lour) Olsson *in vitro*. Plant Physiol **96:** 178–183

Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J **7:** 3315–3320

Deikman J, Hammer PE (1995) Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. Plant Physiol **108:** 47–57

Deikman J, Kline R, Fischer RL (1992) Organization of ripening and ethylene regulatory regions in a fruit-specific promoter from tomato (*Lycopersicon esculentum*). Plant Physiol **100:** 2013–2017

DellaPenna D, Alexander DC, Bennett AB (1986) Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening. Proc Natl Acad Sci USA **83:** 6420–6424

DellaPenna D, Kates DS, Bennett AB (1987) Polygalacturonase gene expression in Rutgers, *rin, nor,* and *Nr*. Plant Physiol **85:** 502–507

DellaPenna D, Lincoln JE, Fischer RL, Bennett AB (1989) Transcriptional analysis of polygalacturonase and other ripening associated genes in Rugters, *rin, nor,* and *Nr* tomato fruit. Plant Physiol **90:** 1372–1377

Giovannoni JJ, DellaPenna D, Bennett AB, Fischer RL (1989) Expression of a chimeric polygalacturonase gene in transgenic *rin* (*ripening inhibitor*) tomato fruit results in polyuronide degradation but not fruit softening. Plant Cell **1:** 53–63

Good X, Kellogg JA, Wagoner W, Langhoff D, Matsumura W, Bestwick RK (1994) Reduced ethylene synthesis by transgenic tomatoes expressing *S*-adenosylmethionine hydrolase. Plant Mol Biol **26:** 781–790

Gray J, Picton S, Shabbeer J, Schuch W, Grierson D (1992) Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Mol Biol **19:** 69–87

Guzman P, Ecker JR (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513–523

Hamilton AJ, Lycett GW, Grierson D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature **346:** 284–287

Holdsworth MJ, Schuch W, Grierson D (1988) Organisation and expression of a wound/ripening-related small multigene family from tomato. Plant Mol Biol **11:** 81–88

Hua J, Chang C, Sun Q, Meyerowitz EM (1995) Ethylene insensitivity conferred by Arabidopsis ERS gene. Science **269:** 1712–1714

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol **44:** 283–307

Kende H, Boller T (1981) Wound ethylene and 1-aminocyclopropane-1-carboxylate synthase in ripening tomato fruit. Planta **151:** 476–481

Kim WT, Yang SF (1992) Turnover of 1-aminocyclopropane-1-carboxylic acid synthase protein in wounded tomato fruit tissue. Plant Physiol **100:** 1126–1131

Klee HJ (1993) Ripening physiology of fruit from transgenic tomato (*Lycopersicon esculentum*) plants with reduced ethylene synthesis. Plant Physiol **102:** 911–916

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J **4:** 403–410

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *Never Ripe* mutation blocks ethylene perception in tomato. Plant Cell **6:** 521–530

Landschulz WH, Johnson PF, McKnight SL (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:** 1759–1764

Li N, Mattoo AK (1994) Deletion of the carboxyl-terminal region of 1-aminocyclopropane-1-carboxylic acid synthase, a key protein in the biosynthesis of ethylene, results in catalytically hyperactive, monomeric enzyme. J Biol Chem **269:** 6908–6917

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit ripening. Proc Natl Acad Sci USA **84:** 2793–2797

Lincoln JE, Fischer RL (1988a) Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet **212:** 71–75

Lincoln JE, Fischer RL (1988b) Regulation of gene expression by ethylene in wild-type and *rin* tomato (*Lycopersicon esculentum*) fruit. Plant Physiol **88:** 370–374

Lizada MCC, Yang SF (1979) A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal Biochem **100:** 140–145

Martin MN, Saftner RA (1995) Purification and characterization of 1-aminocyclopropane-1-carboxylic acid *N*-malonyltransferase from tomato fruit. Plant Physiol **108:** 1241–1249

Montgomery J, Pollard V, Deikman J, Fischer RL (1993) Positive and negative regulatory regions control the spatial distribution of polygalacturonase transcription in tomato fruit pericarp. Plant Cell **5:** 1049–1062

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell **2:** 279–289

Odell JT, Nagy F, Chua N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810–812

Oeller PW, Min-Wong L, Taylor L, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254:** 437–439

Ow DW, Wood KV, DeLuca M, deWet JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science **234:** 856–859

Peck SC, Reinhardt D, Olson DC, Boller T, Kende H (1992) Localization of the ethylene-forming enzyme from tomatoes, 1-aminocyclopropane-1-carboxylate oxidase, in transgenic yeast. J Plant Physiol **140:** 681–686

Peñarrubia L, Aguilar M, Margossian L, Fischer RL (1992) An antisense gene stimulates ethylene hormone production during tomato fruit ripening. Plant Cell **4:** 681–687

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J **3:** 469–481

Prescott AG (1993) A dilemma of dioxygenases (or where biochemistry and molecular biology fail to meet). J Exp Bot **44:** 849–861

Rhodes MJC (1980) The maturation and ripening of fruits. *In* KV Thimann, ed, Senescence in Plants. CRC Press, Boca Raton, FL, pp 157–205

Riov J, Yang SF (1982) Autoinhibition of ethylene production in citrus peel discs. Suppression of 1-aminocyclopropane-1-carboxylic acid synthesis. Plant Physiol **69:** 687–690

Roach PL, Clifton IJ, Fulop V, Harlos K, Barton GJ, Hajdu J, Andersson I, Schofild CJ, Baldwin JE (1995) Crystal structure of isopenicillin *N* synthase is the first from a new structural family of enzymes. Nature **375:** 700–704

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and flower senescence. J Mol Biol **222:** 937–961

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Singh A, Evensen KB, Kao T-H (1992) Ethylene synthesis and floral senescence following compatible and incompatible pollinations in *Petunia inflata*. Plant Physiol **99:** 38–45

Tang X, Gomes AMTR, Bhatia A, Woodson WR (1994) Pistil-specific and ethylene-regulated expression of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. Plant Cell **6:** 1227–1239

Theologis A (1992) One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell **70:** 181–184

Theologis A (1996) Plant hormones: more than one way to detect ethylene. Curr Biol **6:** 144–145

Theologis A, Oeller PW, Wong L-M, Rottmann WH, Gantz DM (1993) Use of a tomato mutant constructed with reverse genetics to study fruit ripening, a complex developmental process. Dev Genet **14:** 282–295

Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, Ryals J (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell **5:** 159–169

Ursin VM, Yamaguchi J, McCormick S (1989) Gametophytic and sporophytic expression of anther-specific genes in developing tomato anthers. Plant Cell **1:** 727–736

van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291–299

Vendrell V, McGlasson WB (1971) Inhibition of ethylene production in banana fruit tissue by ethylene treatment. Aust J Biol Sci **24:** 885–895

Whitehead CS, Fujino DW, Reid MS (1983) Identification of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in pollen. Sci Hortic **21:** 291–297

Wilkinson JQ, Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by *Never-ripe*. Science **270:** 1807–1809

Xu R, Goldman S, Coupe S, Deikman J (1996) Ethylene control of E4 transcription during tomato fruit ripening involves two cooperative *cis*-elements. Plant Mol Biol (in press)

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol **35:** 155–189

Yip W-K, Moore T, Yang SF (1992) Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. Proc Natl Acad Sci USA **89:** 2475–2479

Zarembinski TI, Theologis A (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol Biol **26:** 1579–1597

Zeroni M, Galil J (1976) Autoinhibition of ethylene formation in nonripening stages of the fruit of sycomore Fig (*Ficus sycomorus* L.). Plant Physiol **57:** 647–650

Zhou D, Mattoo AK, Tucker ML (1996) The mRNA for an *ETR1* homolog in tomato is constitutively expressed in vegetative and reproductive tissues. Plant Mol Biol (in press)